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14. ABSTRACT

One of the major challenges in breast cancer research is to develop new chemotherapeutic and chemopreventive agents, particularly for non-estrogen dependent and drug-resistant estrogen dependent breast cancers. SXR activators were able to cause cell cycle arrest and apoptosis in ER^+ and ER^- breast cancer cell lines in culture. Different SXR activators caused accumulation of p53 in ER^+ breast cancer cells leading to increase in its target genes involved in apoptosis and cell cycle. Confirmation of these results in at least two ER^+ breast cancer cell lines suggests validity of this model in estrogen dependent breast cancer. Establishment of loss of function studies in these cell lines confirmed the significance and requirement of SXR in these compounds led apoptosis. Moreover, loss of function studies and microarray studies on estrogen receptor negative cells lines in next year of funding will be able to establish the role of SXR as apoptosis inducer in estrogen receptor independent cells and its mechanism of action in these cell lines. This will provide opportunities for rational drug design and improvement of the efficacy of existing drugs that act through SXR.

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SXR, A Novel Target for Breast Cancer Therapeutics -Verma, Suman

Introduction:

Anti-estrogens such as tamoxifen are important therapeutic agents in the treatment and chemoprevention of breast cancers. Other compounds such as phytoestrogens, fatty acid amides such as anandamide and retinoid X receptor agonists are also effective against breast cancer in cell lines and in animal models. Because these compounds are structurally unrelated, it has not been appreciated that they might act through a common mechanism. All of these compounds share the ability to activate a heterodimer of the steroid and xenobiotic receptor (SXR) and retinoid X receptor (RXR). Our hypothesis is that SXR serves as a common molecular target for some of the anti-proliferative effects of these compounds and that activation of SXR is itself anti-proliferative. To this end, we have found that activation of SXR leads to apoptosis and G1 cell cycle arrest through a p53 dependent pathway in estrogen receptor positive MCF7 breast cancer cells. In this period of study we have been able to establish this pathway in another ER^+ breast cancer cell line, ZR-75-1. We have also been able to show that activation of SXR not only causes increase in p53 mRNA, but also causes stabilization and increased levels of p53 protein in both MCF7 and ZR-75-1 cells. We have performed knockdown experiments and shown that siRNA transfection significantly reduces levels of SXR mRNA and protein in MCF-7 cells. Knocking down of SXR by specific SXR-siRNA but not by scrambled siRNA blocked the induction of iNOS by SXR activators, which confirmed

the role of SXR in induction of iNOS in MCF-7 cells. We have also used real time RT-PCR and specific inhibitors of NOS to confirm that the SXR induced increase in NO is due to increased expression and activity of iNOS and not because of other forms of NOS such as eNOS (endothelial nitric oxide synthase) and nNOS (neuronal nitric oxide synthase). Taken together all these results affirm the role of SXR as an apoptosis inducer in $ER^{+}p53^{wt}$ breast cancer cells. Currently, these results are under review for publication in Molecular Cancer Research.

Our previous results have shown that SXR can induce apoptosis not only in $p53^{wt}$ breast cancer cells but also in $p53^{mut}$ cells. To further extend our study and as a first step in identifying the mechanism by which SXR activators induce apoptosis in $p53^{mut}$ breast cancer cells, we have tested a panel of genes responsible for cell cycle and apoptosis in response to SXR activation.

Body:

Aim#2: What is the molecular pathway underlying the anti-proliferative effects of SXR in ERα^{-}, p53^{mut} cell lines?

2.1 Does SXR loss-of-function inhibit the anti-proliferative effects of SXR activators?

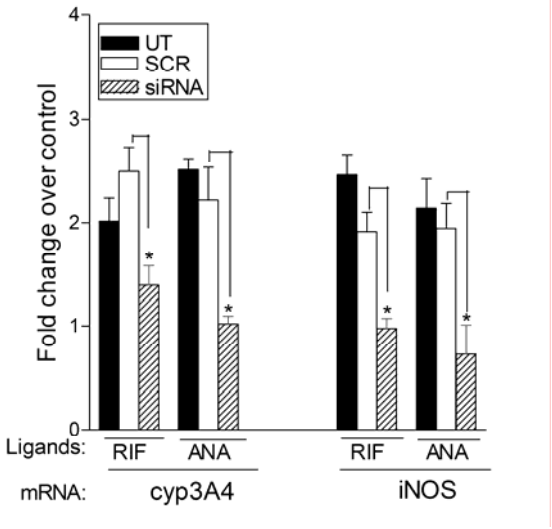

Figure 1 Ligand induced up-regulation of iNOS is directly regulated by SXR. SXR-siRNA but not SCR transfection blocked the ability of SXR ligands (RIF and ANA) to induce up-regulation of CYP3A4 and iNOS. Un-transfected MCF-7 cells, or cells transfected with siRNA or SCR sequence (36 nM each) were treated with 10 μM RIF or ANA for 48 hr. Target gene expression was tested by QRT-PCR. The data are shown as average fold induction relative to solvent control in triplicates ± S.E.M. * *represents P<.05* in comparison to SCR (by student's t test).

In a previous report, we have shown that activation of SXR induces the expression of iNOS. Increased iNOS levels increase the mRNA and protein levels of p53 and causes cell cycle arrest and apoptosis in MCF-7 and ZR-75-1 cells. To confirm the requirement for SXR in the regulation of iNOS expression and iNOS-mediated up-regulation of p53 and its target genes in breast cancer cells, we have investigated the effects of SXR loss-of-function on the SXR ligand-dependent induction of gene expression. As shown in the previous report, treatment with a specific siRNA against SXR, but not with a control scrambled siRNA, reduced the SXR mRNA level by more than 80%, and protein level by more than 60% in MCF-7 cells. For gene expression assays, MCF-7 cells were transfected with siRNA two day prior to ligand treatment, or the day of ligand treatment. After 48 hr of treatment with 10 μM rifampicin and anandamide, RNA was isolated, cDNA synthesized and QRT-PCR

analysis performed. As shown in Figure 1, rifampicin and anandamide induced up-regulation of CYP3A4 and iNOS mRNA levels were diminished by the SXR siRNA (siRNA), but not by scrambled siRNA (SCR). This confirmed the requirement of SXR function to mediate the iNOS and p53-dependent downstream pathways resulting in apoptosis and cell cycle arrest in MCF-7 cells.

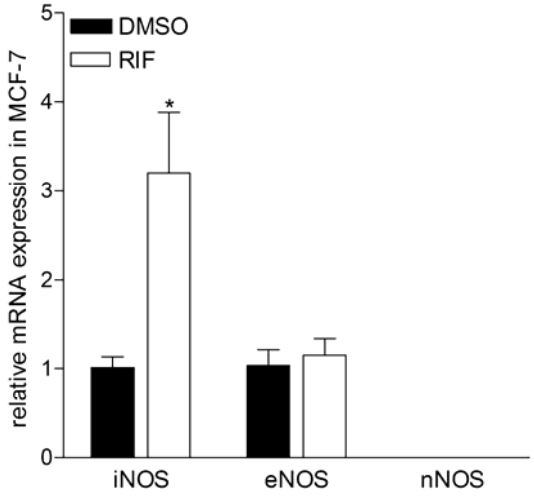

Figure 2: SXR activators cause a specific increase in iNOS expression. Total RNA extracted from MCF-7 cells treated with 10 μM rifampicin for 24 hours was tested for iNOS, eNOS and nNOS expression

Does SXR activation increase NO production specifically through iNOS?

SXR activators induce the expression of iNOS in MCF-7 and ZR-75-1 cells which increases the intra-cellular level of nitric oxide (NO) in these cells. NO can be produced in cells not only by iNOS but also by other forms of NOS such as eNOS (endothelial NOS) and nNOS(neuronal NOS) and also by other intra-cellular processes. To assess whether SXR activation specifically induces NO production via iNOS, we employed two approaches.

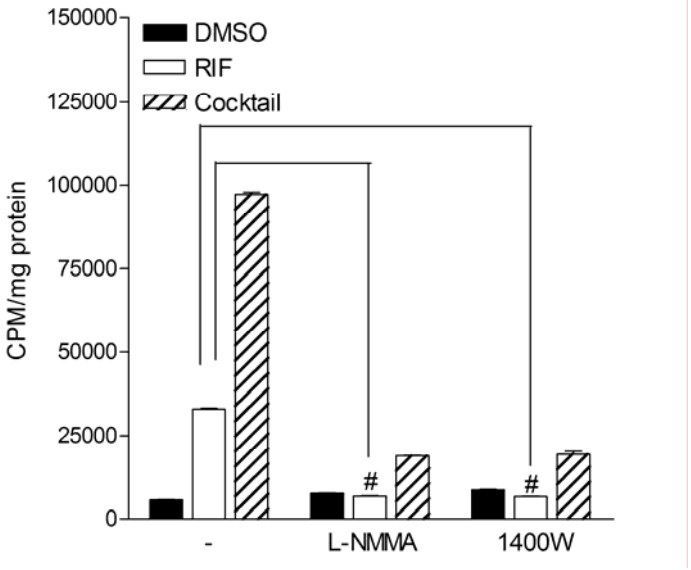

Figure 3: SXR activation increases iNOS activity. MCF-7 cells were activated by rifampicin or solvent control for 24 and 48 hours. Cytokine cocktail (IL1β (20 ng/ml), TNFα(15 ng/ml) and LPS(1 mg/ml) was used as a positive control . Nitric oxide synthase activity was determined in total cells homogenates in the presence or absence of NOS inhibitors; L-NMMA and 1400W (10 μM). The data are depicted as counts per minute (CPM) per mg protein. * *represents P<.001* in comparison to RIF (by student's t test)

First, we evaluated the changes in the mRNA levels of all three form of NOS in response to SXR activation by QRT-PCR. As shown in Figure 2, rifampicin treatment caused a selective increase in iNOS mRNA level but not in eNOS. In agreement with previous reports, we did not detect basal or induced expression of nNOS in MCF-7 cells (1, 2).

In the second approach, we tested NOS activity in the homogenates made from MCF-7 cells treated with 10μM rifampicin in the presence or absence of specific iNOS inhibitor "1400W" or a non-specific NOS inhibitor "L-NMMA" (L-N(G)-monomethylarginine) . MCF-7 cells were treated with 10 μM rifampicin or DMSO for 24 and 48 hrs. The cells were also treated with a cocktail of IL-1β (20 ng/ml), TNFα (15 ng/ml) and LPS (1 mg/ml) as a positive control for iNOS induction. After the indicated time periods, cell lysates were made in 1X homogenization buffer (25 mM Tris-Hcl pH7.4, 1 mM EDTA, 1 mM EGTA) by sonicating cells twice for 10s at 10 amp. The NOS activity was detected by measuring the conversion of ^{14}C L-arginine (Amersham Bioscience,USA) in to ^{14}C L-citrulline by using nitric oxide synthase assay kit (Calbiochem Inc., USA). The radioactivity was measured in a scintillation counter and the counts were adjusted for per mg protein (CPM/mg). MCF-7 cells treated with rifampicin elicited a significant increase in NOS activity (Figure 3). As expected, treatment with a cytokine cocktail also caused a significant increase in NOS activity (3). The increased NOS activity by either rifampicin or by cytokine cocktail treatment could be completely blocked by either a non-specific NOS inhibitor, L-NMMA or the iNOS specific inhibitor 1400W (Figure 3). Together, these results confirm that the increase in NOS activity in MCF7 cells resulting from treatment with SXR activators is primarily due to increased iNOS levels and activity.

Does SXR activation cause p53 stabilization and accumulation?

In the previous report we have shown that SXR activation induces stabilization and accumulation of p53 in MCF-7 cells. In this period of study we further confirmed these results in another ER^+ breast cancer cell line (ZR-75-1). Cell lysates were made from ZR-75-1 cells treated with SXR activators rifampicin (RIF), tamoxifen(TAM), anandamide(ANA) and clotrimazole (CLO) for 24 hours and subjected to western blot analysis using anti-p53 antibody (FL-393 HRP, Santa Cruz Biotechnology Inc., USA). Camptothecin (CAMP) was used as a positive control for p53 induction. The blot was stripped and re-probed with GAPDH anti-body (Ambion Inc., USA) as a loading control. Chemiluminescence was assayed using an Alpha Innotech Fluorchem SP imager (Alpha Innotech Inc., CA,USA) and analyzed by FluorChem AlphaEase FC software (Alpha Innotech). Similar to the effects seen in MCF-7 cells, all four SXR activators resulted in increased p53 protein levels in ZR-75-1 cells (Figure 4). These results confirm that activation of SXR leads to increased levels of p53 and that this is a common mechanism for SXR activator-induced apoptosis in ER^+p53^{wt} breast cancer cell lines.

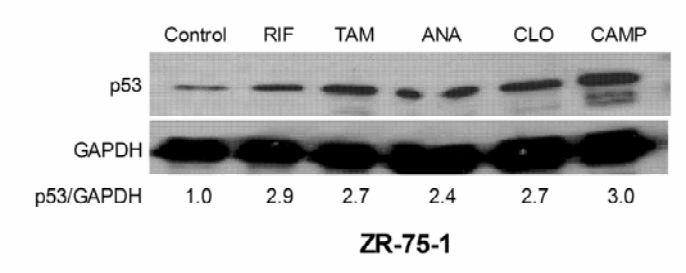

Figure 4: Cell lysates made from ZR-75-1 cells treated with SXR agonists (10 μM) or solvent controls for 24 hours were subjected to Western blot analysis using p53 antibody (FL-393 HRP, Santa Cruz Inc.). Equal loading was confirmed by stripping and re-probing the same blot with an anti-GAPDH antibody (mouse monoclonal 6C5, Ambion, Inc., Austin, TX). camptothecin (CAMP) was used as a positive control for p53 induction. The bands were analyzed by FluorChem AlphaEase FC software (Alpha Innotech). Note the gap in ANA lane is because of gel tearing at the time of transferring.

2.3 Which target genes mediate the anti-proliferative effects of activated SXR in ER$^-$ cell lines?

Literature search and testing NF-kB target genes in ER$^-$ cells:

Our previous results have shown that SXR activators induce apoptosis and G2/M cell cycle arrest in ER$^-$p53^{mut} breast cancer cells (MDA-MB-435 and MDA-MB-231). Since these cells are mutated in p53, we did not expect the observed apoptosis to be through p53 mediated pathway as shown in MCF-7 cells. Previously we have reported that there is a negative cross-talk between SXR and NF-κB (4). Based on these observations, we hypothesized that the decreased proliferation from SXR activators that we are seeing in ER$^-$ cell lines results from inhibition of NF-κB. Both ER$^-$ cell lines that we studied in proliferation assay (MDA-MB-231 & MDA-MB-435) are known to have aberrantly

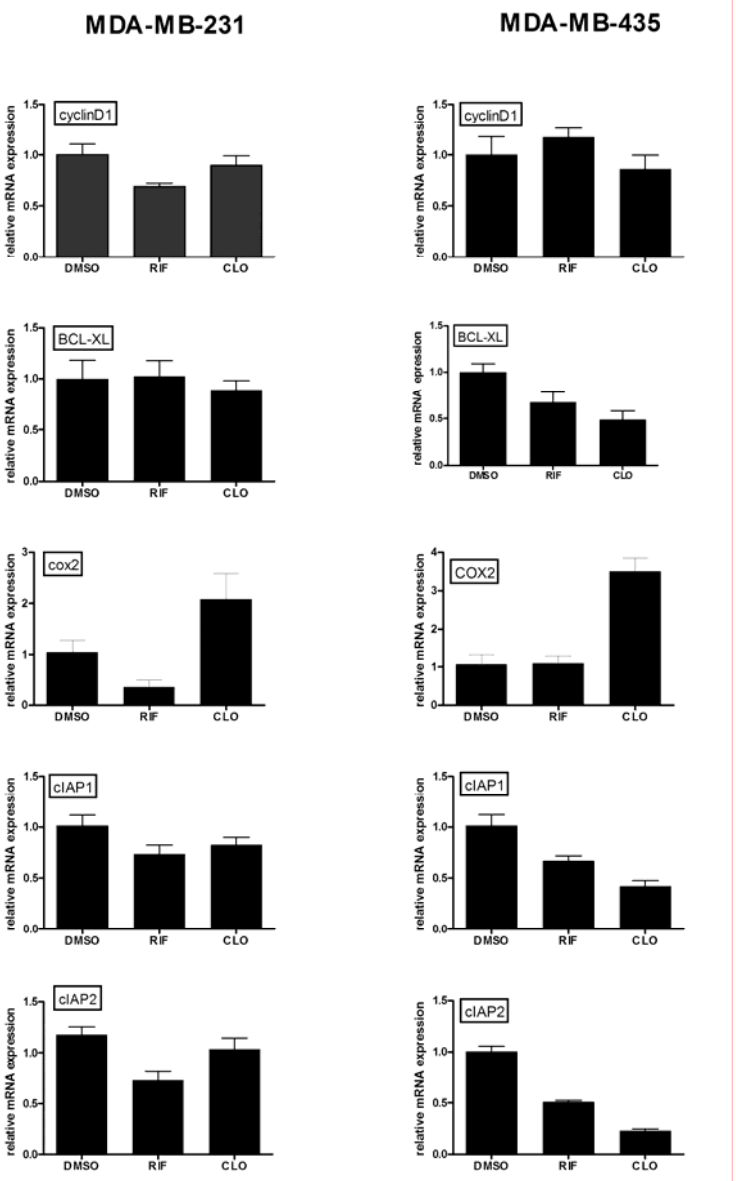

Figure 5: Gene expression changes in response to SXR activation in ER$^-$ breast cancer cell lines: MDA-MB-435 and MDA-MB-231 cells were treated with 10μM rifampicin (RIF) and anadamide (ANA) for 24 hours. QRT-PCR was performed using primers for NF-kB target genes: CyclinD1, BCL-XL, Cox2, cIAP1, cIAP2.

active NF-κB (5), and inhibition of NF-κB in these cell lines leads to apoptosis (6-8). Therefore, in the process of finding the genes responsible for decreased proliferation of $ER^{-}p53^{mut}$ cells by SXR activators, we decided to first look at the NF-κB target genes which are involved in cell cycle or apoptosis such as CyclinD1, Bcl-XL, COX2, cIAP1 & cIAP2. MDA-MB-231 and MDA-MB-435 cells were treated with SXR activators-rifampicin and clotrimazole for 24 & 48 hours. Total RNA was isolated after the indicated time period using Trizol reagent. After cDNA synthesis using superscriptIII (Invitrogen inc., USA), gene expression changes were measured in comparison to DMSO control by QRT-PCR. As shown in Figure 5 we did not observe any consistent changes in the gene expression level of any one of the tested genes by both SXR activators in both breast cancer cell lines at any time point. As we did not see any changes in the genes that we expected to change by SXR activator treatment next we are planning to perform GEArray Focused DNA Microarray (SuperArray Bioscience corp., USA) . These GEArrays are pre-printed arrays with set of relevant pathway focused genes. As we already know that SXR activators induce caspase dependent apoptosis and G2/M cell cycle arrest in $ER^{-}p53^{mut}$ breast cancer cells, we will perform apoptosis and cell cycle pathway focused microarrays first and then based on these results we can further evaluate the mechanism responsible for changes in apoptosis or cell cycle genes. In a pathway focused approach, we should be able to delineate the mechanism responsible for the induction of apoptosis by SXR activators in a quicker manner.

Key Research Accomplishments:

- Confirmed the requirement of SXR in induction of iNOS by SXR ligands using gene knock down study.

- Confirmed that SXR activators led increase in NO levels in breast cancer cells is specifically through iNOS.

- Confirmed that SXR ligands cause accumulation of p53 in $ER^{+}p53^{wt}$ breast cancer cell.

- Tested a panel of NF-κb target genes in response to SXR activation in MDA-MB-231 and MDA-MB-435 cells.

Reportable Outcomes:

Submitted a revised manuscript to Molecular Cancer Research (MCR_07_2091R). It is currently under review.

Presentations:

October 19, 2007: Seminar presentation at Meeting of Los Angeles Area Receptor Labs (MOLAR), City of Hope, Duarte, CA

November 15, 2007: Seminar presentation at Meeting for Research in Progress held at UCI, Irvine, CA

December 19, 2007: Seminar presentation at Jawahar Lal Nehru University, New Delhi, India

Conclusions:

One of the major challenges in breast cancer research is to develop new chemotherapeutic and chemopreventive agents, particularly for non-estrogen dependent and drug-resistant estrogen dependent breast cancers. SXR activators were able to cause cell cycle arrest and apoptosis in ER^+ and ER^- breast cancer cell lines in culture. Different SXR activators caused accumulation of p53 in ER^+ breast cancer cells leading to increase in its target genes involved in apoptosis and cell cycle. Confirmation of these results in at least two ER^+ breast cancer cell lines suggests validity of this model in estrogen dependent breast cancer. Establishment of loss of function studies in these cell lines confirmed the significance and requirement of SXR in these compounds led apoptosis. Moreover, loss of function studies and microarray studies on estrogen receptor negative cells lines in next year of funding will be able to establish the role of SXR as apoptosis inducer in estrogen receptor independent cells and its mechanism of action in these cell lines. This will provide opportunities for rational drug design and improvement of the efficacy of existing drugs that act through SXR.

References:

1. Loibl S, Bratengeier J, Farines V*, et al.* Investigations on the inducible and endothelial nitric oxide synthases in human breast cancer cell line MCF-7 - estrogen has an influence on e-NOS, but not on i-NOS. Pathology - Research and Practice 2006;202(1):1-7.

2. Mortensen K, Skouv J, Hougaard DM, Larsson LI. Endogenous endothelial cell nitric-oxide synthase modulates apoptosis in cultured breast cancer cells and is transcriptionally regulated by p53. The Journal of biological chemistry 1999;274(53):37679-84.

3. Binder C, Schulz M, Hiddemann W, Oellerich M. Induction of inducible nitric oxide synthase is an essential part of tumor necrosis factor-alpha-induced apoptosis in MCF-7 and other epithelial tumor cells. Laboratory investigation; a journal of technical methods and pathology 1999;79(12):1703-12.

4. Zhou C, Tabb MM, Nelson EL*, et al.* Mutual repression between steroid and xenobiotic receptor and NF-kappaB signaling pathways links xenobiotic metabolism and inflammation. The Journal of clinical investigation 2006;116(8):2280-9.

5. Nakshatri H, Bhat-Nakshatri P, Martin DA, Goulet RJ, Jr., Sledge GW, Jr. Constitutive activation of NF-kappaB during progression of breast cancer to hormone-independent growth. Mol Cell Biol 1997;17(7):3629-39.

6. Biswas DK, Shi Q, Baily S*, et al.* NF-kappa B activation in human breast cancer specimens and its role in cell proliferation and apoptosis. Proc Natl Acad Sci U S A 2004;101(27):10137-42.

7. Biswas DK, Dai SC, Cruz A, Weiser B, Graner E, Pardee AB. The nuclear factor kappa B (NF-kappa B): a potential therapeutic target for estrogen receptor negative breast cancers. Proc Natl Acad Sci U S A 2001;98(18):10386-91.

8. Monks NR, Biswas DK, Pardee AB. Blocking anti-apoptosis as a strategy for cancer chemotherapy: NF-kappaB as a target. J Cell Biochem 2004;92(4):646-50.